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K^+ STIMULATION OF ADP/ATP EXCHANGE CATALYZED BY THE (Na^+ + K^+)-DEPENDENT ATPase

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Summary

A (Na^+ + K^+)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparation from rat brain catalyzed a Na^+-dependent ADP/ATP exchange reaction. With millimolar concentrations of ATP and ADP, successively higher concentrations of $MgCl_2$ inhibited the exchange reaction. Examination of this inhibition in terms of the concentrations of free Mg^{2+} and Mg · nucleotide complexes indicated that inhibition resulted from either Mg^{2+} or Mg · nucleotide occupying the low-affinity substrate sites of the enzyme (K_m for Mg · ATP about 0.5 mM); earlier studies assigned catalysis of the exchange reaction to the high-affinity substrate sites (K_m for Mg · ATP about 1 μM). When inhibition could be attributed to occupancy of the low-affinity sites by Mg · ATP, K^+ stimulated ADP/ATP exchange. The sites through which K^+ relieved inhibition due to Mg · ATP appeared to be the moderate-affinity α-sites for K^+, as indicated by the concentration dependence, the effect of dimethyl sulfoxide on apparent affinity for K^+, and by the inability of Li^+ to substitute for K^+. The observations fit an earlier formulation of antagonism between K^+ at the α-sites and Mg · ATP at the low-affinity substrates sites. Under conditions in which inhibition of ADP/ATP exchange could be attributed instead to Mg^{2+} occupying the low-affinity substrate sites, K^+ produced little or no stimulation, in accord with earlier studies showing that K^+ at the α-sites is a poor antagonist toward Mg^{2+}. With micromolar concentrations of ATP and ADP, K^+ inhibited the exchange reaction, and competition toward Na^+ at the Na^+-sites was apparent.

Introduction

The (Na^+ + K^+)-dependent ATPase (EC 3.6.1.3) catalyzes a Na^+-dependent ADP/ATP exchange reaction [1—3]. Current formulations for the ATPase reaction sequence depict a Na^+-dependent phosphorylation of the enzyme that is

followed by a K^+-dependent dephosphorylation [3]:

$$E \underset{}{\overset{Mg \cdot ATP, Na^+}{\rightleftharpoons}} E - P \xrightarrow{K^+} E + P_i$$

Since the exchange reaction has been interpreted to be a reversal over the initial step of the overall reaction sequence [1], the addition of K^+ would be expected to inhibit ADP/ATP exchange by pulling the reaction to the right. Nevertheless, Banerjee and Wong [4] described a stimulation of Na^+-dependent ADP/ATP exchange by K^+.

This paper is concerned with the mechanism by which K^+ stimulates the exchange reaction. Recent experiments indicate that there are two classes of substrate sites for the ATPase [2,5—9]: high-affinity sites (K_m for MgATP of about 1 μM [8]), and low-affinity sites (K_m for Mg · ATP of about 0.5 mM [8]). The exchange reaction appears to be catalyzed by the high-affinity class of sites, whereas occupancy of the low-affinity sites by Mg · ATP, or free Mg^{2+}, results in inhibition of the exchange reaction [2]. Since K^+ reduces the apparent affinity for Mg · ATP at the low-affinity sites [10,11] it could thus indirectly stimulate ADP/ATP exchange by reducing the binding of inhibitory Mg · ATP. Experiments to test this hypothesis are described here.

Methods and Materials

The enzyme preparation was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described [11].

ADP/ATP exchange activity was measured in terms of the incorporation of ^{14}C into ATP during incubations at 37° C with unlabeled ATP and [U-^{14}C]ADP (about 500 000 cpm) [2]. Incubation media contained 30 mM histidine · HCl/Tris (pH 7.8), the concentrations of ADP, ATP (both as Tris salts), and $MgCl_2$ indicated, plus either NaCl or 0.1 mM ouabain. The final volume was 50 μl. The reaction was terminated by placing the incubation tubes in a boiling water bath for 2 min [1]. To separate ATP, ADP, and AMP, a 20 μl portion of the reaction mixture, with carrier unlabeled nucleotides added, was spotted on washed polyethyleneimine cellulose-coated plastic films (Brinkman Instrument Co.) for thin-layer chromatography in 1.2 M LiCl [2]. The nucleotides were located on the developed films by their absorption of ultraviolet light, and these regions were cut from the films for liquid scintillation counting. The rate of incorporation of ^{14}C into ATP measured in the presence of NaCl, less the rate in the presence of ouabain, was considered to be the Na^+-dependent ADP/ATP exchange rate [1,2].

Total ATPase activity was measured in terms of the production of [^{14}C]ADP in parallel incubations with [U-^{14}C]ATP and unlabeled ADP [2].

[U-^{14}C]ADP (500—600 Ci/mol) and [U-^{14}C]ATP (500—600 Ci/mol) were purchased from Amersham-Searle. Unlabeled ATP, ADP, and AMP were purchased from Sigma.

Data presented are averages of five or more experiments, each performed in duplicate.

Results and Discussion

Inhibition of exchange activity by $MgCl_2$

The ($Na^+ + K^+$)-dependent ATPase preparation catalyzed a Na^+-dependent ADP/ATP exchange, which, in the presence of 1 mM ADP, ATP, and $MgCl_2$, and 20 mM NaCl, averaged 30 μmol [^{14}C]ATP formed/min · mg protein. For ease of comparison the rate of exchange activity under these conditions was assigned a relative value of 1.0, and rates under other conditions, measured concurrently, are expressed relative to this value.

With these experimental conditions adenylate kinase activity was not detectable: there was no measurable formation of [^{14}C]AMP. Total ATPase activity, representing the Na^+-dependent ATPase activity of the ($Na^+ + K^+$)-dependent ATPase [3] plus any contaminating Mg^{2+}-ATPase activity, was present at a level comparable to that of the exchange activity. Incubation times were chosen so that metabolism of either ATP or ADP, by all routes, was less than 10 per cent of the initial concentration; under these conditions the production of [^{14}C]ATP was linear with time.

Na^+-dependent ADP/ATP exchange decreased as the concentration of $MgCl_2$ was successively raised (Table I), as previously described [1,2]. But when the concentrations of free Mg^{2+} and Mg · nucleotide complexes are calculated, from dissociation constants measured in this medium [2,12], no simple relationship to inhibition was observed (Table I). For example, when ATP was present at a high concentration, 5 mM, successively higher levels of $MgCl_2$ produced inhibition that could be correlated with higher concentrations of the Mg · ATP complex. On the other hand, when ATP was present at a low concentration,

TABLE I

EFFECT OF KCl ON Na^+-DEPENDENT ADP/ATP EXCHANGE ACTIVITY

Na^+-dependent ADP/ATP exchange was determined from incubations with 20 mM NaCl, 1 mM ADP, and the concentrations of ATP and $MgCl_2$ listed, as described under Methods and Materials; velocities are expressed relative to that from incubations with 1 mM ATP and $MgCl_2$ defined as 1.0, ±S.E.M. The concentration of KCl, when added, was 10 mM. Concentrations of free Mg^{2+} and the Mg-nucleotide complexes were calculated using the following values for the dissociation constants: for Mg · ATP, 32 μM [2], and for Mg · ADP, 360 μM [2].

Concentrations of additions (mM)		Calculated concentrations of reactants (mM)			Relatively velocity		Per cent change
ATP	$MgCl_2$	Mg^{2+}	Mg · ATP	Mg · ADP	no KCl	with KCl	
0.2	0.2	0.029	0.096	0.075	0.58 ± 0.04	0.57 ± 0.05	−2
0.2	1.0	0.336	0.182	0.482	0.51 ± 0.04	0.68 ± 0.03	33
0.2	2.0	1.060	0.194	0.746	0.38 ± 0.03	0.48 ± 0.04	26
1.0	0.2	0.007	0.175	0.018	1.71 ± 0.12	2.14 ± 0.16	25
1.0	1.0	0.085	0.725	0.190	1.00	2.66 ± 0.12	166
1.0	2.0	0.487	0.938	0.575	0.56 ± 0.03	1.90 ± 0.13	239
1.0	3.0	1.249	0.975	0.776	0.31 ± 0.04	0.93 ± 0.09	200
5.0	0.2	0.002	0.194	0.004	1.48 ± 0.10	2.04 ± 0.14	38
5.0	1.0	0.008	0.971	0.021	0.68 ± 0.06	2.52 ± 0.18	271
5.0	2.0	0.020	1.927	0.053	0.18 ± 0.03	0.75 ± 0.13	317
5.0	3.0	0.042	2.852	0.106	0.06 ± 0.03	0.19 ± 0.04	220

0.2 mM, successively higher levels of $MgCl_2$ produced inhibition that could be correlated with higher concentrations of free Mg^{2+}. In this latter case, relatively little inhibition was seen with a concentration of free Mg^{2+}, 0.3 mM, that was far greater than the free Mg^{2+} present (0.05 mM) in the experiments with 5 mM ATP and 3 mM $MgCl_2$, where the inhibition was almost complete. Conversely, in experiments with 0.2 mM ATP, marked inhibition occurred with a concentration of Mg · ATP, 0.2 mM, that was unassociated with inhibition in experiments with 5 mM ATP and 0.2 mM $MgCl_2$. Inhibition, therefore, appears to be associated with increased levels of either free Mg^{2+} or Mg · nucleotide.

These observations are in accord with recent experiments on ADP/ATP exchange activity of an eel electric organ ATPase preparation [2]: inhibition became apparent when the concentration of either Mg^{2+} or Mg · ATP was increased into the range 0.3—1 mM. (The data suggested that Mg · ADP, in this same concentration range, may also inhibit the exchange reaction.)

The inhibitory concentrations, seen here and in the previous experiments, are in reasonable correspondence with estimated affinities for all these substances at the low-affinity substrate sites of this ATPase preparation [8,10,13]: the K_m for Mg · ATP and free Mg^{2+} are 0.5 and 0.8 mM, respectively; the K_i for Mg · ADP toward Mg · ATP is 0.3 mM. On the other hand, the exchange reaction itself appears to be catalyzed at the high-affinity substrate sites [2], which have a K_m for Mg · ATP of about 0.001 mM [8].

Stimulation of exchange activity by KCl

If inhibition by $MgCl_2$ is due, at least in certain circumstances, to formation of the Mg · ATP complex, which subsequently occupies low-affinity substrate sites inhibitory to the exchange reaction, then a mechanism by which K^+ can stimulate exchange becomes apparent. In kinetic studies on the overall (Na^+ + K^+)-dependent ATPase [10,11], K^+ decreases the apparent affinity for Mg · ATP at the low-affinity substrate sites. K^+ could thus relieve inhibition of the exchange reaction by antogonizing the occupancy of the low-affinity substrate sites by Mg · ATP. (In these experiments the concentrations of Mg · ATP and Mg · ADP are far greater than the K_m values for the high-affinity substrate sites that catalyze the exchange reaction: the catalytic sites thus would be nearly saturated even with binding there also antagonized by K^+.)

If Mg · ATP acts as a noncompetitive inhibitor to the exchange reaction, and K^+ antagonizes the binding to the inhibitory sites, then the percentage increase in activity due to K^+ should increase with the concentration of inhibitor, Mg · ATP. This was observed (Table I).

By contrast, when inhibition was associated chiefly with increased levels of free Mg^{2+} (concentrations above 0.2 mM), as in experiments with 0.2 mM ATP, then stimulation due to K^+ was far less. This lesser effect is in accord with the weak antagonism observed between Mg^{2+} at the low-affinity substrate sites and K^+ [13].

The sites at which K^+ acts to antagonize the binding of Mg · ATP are the "α-sites" [10,13], moderate-affinity sites for K^+. These α-sites can be distinguished from "β-sites", high-affinity sites through which K^+ activates the hydrolysis of the enzyme-phosphate intermediate in the overall ATPase reaction, by several criteria, including apparent affinity for K^+ and the effect of

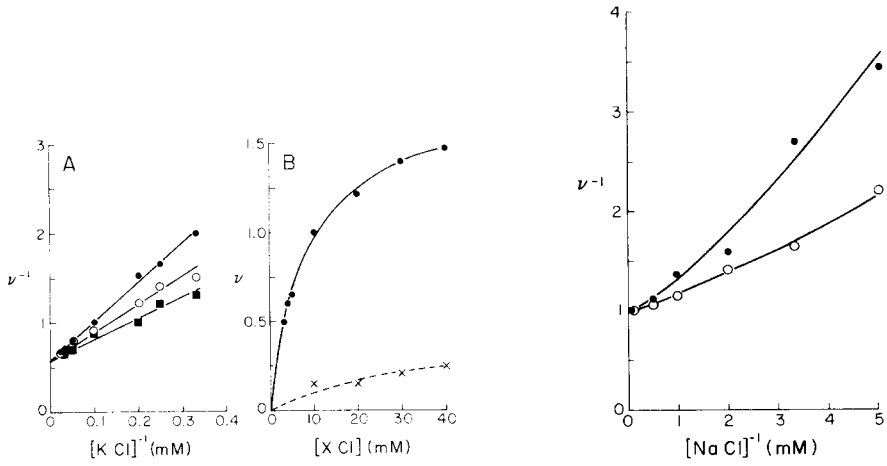

Fig. 1. Effect of KCl and LiCl on Na^+-dependent ADP/ATP exchange. In panel A exchange was measured from incubations with 1 mM ATP, ADP, and $MgCl_2$, with the concentrations of KCl indicated and either 20 mM NaCl (•), 10 mM NaCl (○), or 20 mM NaCl in the presence of 10% (v/v) dimethyl sulfoxide (■). Data are presented in terms of the K^+-stimulated increment in exchange activity with 20 mM NaCl and 10 mM KCl, defined as 1.0, in Lineweaver-Burk form. In panel B the effect on ADP/ATP exchange of LiCl (X) is compared to that of KCl (•), in the presence of 1 mM ATP, ADP, and $MgCl_2$, and 20 mM NaCl. Data are presented relative to the increment in exchange activity due to 10 mM KCl.

Fig. 2. Effect of NaCl on Na^+-dependent ADP/ATP exchange. Exchange activity was measured in the presence of 1 mM ATP, ADP, and $MgCl_2$, with the concentrations of NaCl indicated, in the absence (•) or presence (○) of 10% dimethyl sulfoxide. Velocities are expressed relative to that with 20 mM NaCl, in the absence of dimethyl sulfoxide, defined as 1.0; data are presented in Lineweaver-Burk form.

dimethyl sulfoxide on this affinity, as well as the relative efficacy of Li^+ as a substitute for K^+ [13].

In the presence of 20 mM NaCl the $K_{0.5}$ for the K^+-dependent increment in exchange activity was 7.7 mM (Fig. 1), whereas with 10 mM NaCl the $K_{0.5}$ was 5.5 mM. These values are in good agreement with the $K_{0.5}$ for K^+ at the α-sites in the absence of Na^+, 2 mM, since the K_i for Na^+ at those sites is 6 mM [14].

Further support for the identification of the sites through which K^+ stimulates the exchange reaction with the α-sites is afforded by two other observations. (i) Dimethyl sulfoxide decreased the $K_{0.5}$ for K^+ at the sites mediating the stimulation of the exchange reaction (Fig. 1), and dimethyl sulfoxide decreases the $K_{0.5}$ for K^+ at the α-sites, but not at the β-sites [13]. (ii) LiCl, even at high concentrations, was a poor substitute for KCl (Fig. 1), and LiCl is far less effective at the α-sites than the β-sites [13].

The Na^+-dependence of the ADP/ATP exchange was also examined, for comparison (Fig. 2). In the absence of K^+ the $K_{0.5}$ for Na^+ was 0.4 mM, in good agreement with the $K_{0.5}$ for the overall ATPase reaction when extrapolated to the absence of K^+, 0.3 mM [14]. In the presence of dimethyl sulfoxide the $K_{0.5}$ for Na^+ was nearly halved (Fig. 2), as with the $K_{0.5}$ for Na^+ for the ATPase reaction [14].

Effect of KCl at lower concentrations of ATP and ADP

If stimulation of ADP/ATP exchange by K^+ is due to K^+ antagonizing the

TABLE II

EFFECT OF KCl ON Na^+-DEPENDENT ADP/ATP EXCHANGE ACTIVITY WITH 50 μM ADP AND ATP

Na^+-dependent ADP/ATP exchange was determined from incubations with 20 mM NaCl, 50 μM ADP, 50 μM ATP, and the concentrations of $MgCl_2$ listed, as in Table I; velocities are expressed relative to that from incubations with 50 μM $MgCl_2$ defined as 1.0, ±S.E.M. The concentration of KCl, when added, was 10 mM. Concentrations of free Mg^{2+} and Mg-nucleotide complexes were calculated as in Table I.

Concentration of $MgCl_2$ (μM)	Calculated concentrations of reactants (μM)			Relative velocity	
	Mg^{2+}	Mg · ATP	Mg · ADP	no KCl	with KCl
50	25	22	3	1.00	0.96 ± 0.07
100	60	33	7	0.89 ± 0.10	0.93 ± 0.06
200	145	41	14	0.82 ± 0.06	0.80 ± 0.03
500	426	47	27	0.44 ± 0.05	0.37 ± 0.04
1000	916	48	36	0.16 ± 0.04	0.17 ± 0.02

binding of Mg · ATP (and/or Mg · ADP) to the low-affinity substrate sites, then negligible stimulation would be expected in experiments where the total ATP (and ADP) concentration was well below the K_m for Mg · ATP (and Mg · ADP) at those sites. In experiments with 50 μM ATP and ADP, successively higher concentrations of $MgCl_2$ again inhibited the exchange reaction (Table II), consistent with free Mg^{2+} occupying the low-affinity substrate sites. But, in accord with the observation that K^+ is a relatively poor antagonist toward Mg^{2+} at these sites [13], no stimulation of exchange activity by K^+ was seen (Table II).

These experiments also permit an investigation of possible interactions between K^+ and Na^+ at the Na^+-sites. In the absence of KCl, Na^+-activation of the exchange reaction with 50 μM ATP and ADP was not distinguishable from that with 1 mM ATP and ADP. With the low concentration of nucleotides, however, inhibition by KCl could be shown (Fig. 3): KCl acted as a competitor

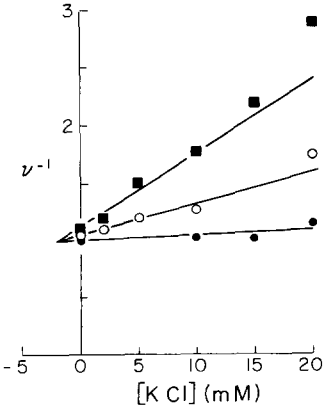

Fig. 3. Inhibition of Na^+-dependent ADP/ATP exchange by KCl. Exchange activity was measured in the presence of 50 μM ATP, ADP, and $MgCl_2$, in the presence of 20 mM (●), 4 mM (○), or 2 mM (■) NaCl, and the concentrations of KCl indicated. Data are presented in the form of a Dixon plot, with velocities expressed relative to that with 20 mM NaCl and no KCl, defined as 1.0.

toward Na^+ at the Na^+-sites, with a K_i of 2 mM. This value is in reasonable accord with the K_i for K^+ at the Na^+-sites of the overall ATPase reaction, 1 mM [14].

These values for the $K_{0.5}$ for Na^+ and K_i for K^+ at the Na^+-sites also indicate that negligible competition would be expected in the presence of 20 mM NaCl, as in the previous experiments with millimolar concentrations of ATP and ADP that showed stimulation of the exchange reaction by KCl. With high NaCl concentrations the stimulatory effects of K^+ at the α-sites would predominate.

Despite the fit of these observations to the kinetic model, at least one additional mode of inhibition by K^+ would seem likely in all the experiments. This is inhibition of the exchange reaction due to K^+ occupying the high-affinity β-sites, through which enzyme dephosphorylation is activated. K^+ at the β-sites should diminish the steady-state level of the enzyme-phosphate intermediate, and hence the rate of exchange. Such an effect could not be distinguished here in the presence of the other actions of K^+, but clearly neither a firm evaluation of the kinetic parameters nor a final delineation of possible intermediary steps is now possible. Nevertheless, the observations do permit a formulation for an unexpected stimulation of the ADP/ATP exchange reaction by K^+, in terms of current models of cation and nucleotide sites of the enzyme.

Conclusions

Stimulation of Na^+-dependent ADP/ATP exchange activity by KCl could be observed under conditions in which exchange activity appeared to be inhibited by Mg · ATP occupying the low-affinity substrate sites of the enzyme. K^+ thus appeared to stimulate by relieving inhibition, consistent with the previously demonstrated antagonism by K^+ toward the binding of Mg · ATP. Moreover, the sites through which K^+ stimulated the exchange reaction were similar, by several criteria, to the α-sites for K^+, through which K^+ antagonizes binding of Mg · ATP at the low-affinity substrate sites. When inhibition was associated with high levels of free Mg^{2+}, rather than the Mg · ATP complex, K^+ produced little or no stimulation, consistent with the lesser antagonism by K^+ toward the binding of Mg^{2+}. In these circumstances competition by K^+ toward Na^+ at the Na^+-sites could be demonstrated. These observations not only can account for the paradoxical stimulation of the ADP/ATP exchange reaction by K^+, but also can be incorporated into formulations of cation and nucleotide interactions in the overall (Na^+ + K^+)-dependent ATPase reaction sequence.

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